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(54) Title: ANTISENSE INHIBITION OF LAMININ-8 EXPRESSION TO INHIBIT HUMAN GLIOMAS

(57) Abstract: Using gene array technology, we observed that an increase of the $\alpha 4$ chain- containing Laminin-8 correlated with poor prognosis for patients with brain gliomas. We established that inhibition of Laminin-8 expression by a new generation of highly specific and stable antisense oligonucleotides (*Morpholino*TM) against chains of Laminin-8 could slow or stop the spread of glioma. This was demonstrated in an *in vitro* model using human glioblastoma multiforme cell lines M059K and U-87MG co-cultured with normal human brain microvascular endothelial cells (HBMVEC). Using Western blot analysis and immunohistochemistry, we confirmed that antisense treatment effectively blocked laminin-8 protein synthesis. Antisense oligonucleotides against both $\alpha 4$ and $\beta 1$ chains of laminin-8 blocked significantly the invasion of co-cultures through Matrigel. The results show that Laminin-8 may not only contribute to glioma progression and recurrence as part of the neovascularization process but also by directly increasing the invasive potential of tumor cells.



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Antisense inhibition of Laminin-8 expression to inhibit human gliomas

The current application is based on and claims priority from U.S.

5 Provisional Patent Application 60/502,729, filed on September 12, 2003.

Introduction

Glial tumors are the leading cause of cancer death in children [1]. Overall, they account for 1.4% of all cancers and 2.4% of all cancer deaths. Average survival time for low-grade astrocytoma or oligodendroglioma patients is 6 to 8
10 years. It decreases to 3 years for patients with anaplastic astrocytoma and drops to 12-18 months for glioblastoma multiforme (GBM). Currently, these tumors are treated by surgical removal, radiation therapy, chemotherapy or combinations of these treatments. The majority of GBMs is highly invasive and rapidly develops recurrences at the primary site. Tumor prognoses and responses to therapy can
15 vary greatly even with the same histological diagnosis [2]. It is generally recognized that the improvement of prognosis, prediction of response to treatment, and development of novel effective therapeutic approaches for glial tumors may largely depend upon the introduction into clinical practice of novel specific markers involved in the development of different gliomas and their
20 subsequent recurrences.

Attempts have been made to establish and characterize a number of glioma markers, such as glial fibrillary acidic protein, vimentin, synaptophysin, and nestin. Determination of differential expression of these markers (immunophenotyping) in gliomas, however, has thus far not altered existing
25 therapeutic approaches, treatment success rates, or disease outcome prediction [2, 3]. Researchers next sought to identify novel glioma markers using powerful gene array technology [4-7]. Recently, our group described a new molecular marker of glial tumors, laminin-8, that was differentially expressed in malignant tumors compared to benign tumors and normal brain tissues [5].

All laminins consist of three covalently linked chains, α , β and γ . To date, 15 members (isoforms) of this family that are present in different basement membranes (BMs) have been described [8-10]. Laminins interact with cells through various receptors. Most of these receptors belong to the family of
5 integrin heterodimers, although other molecules including dystroglycan complex and Lutheran blood group glycoprotein have also been shown to bind to laminins. In different cell types, integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$ and $\alpha_7\beta_1$ have been reported to have the capability to bind to laminins. Specific laminin isoforms bind some but not all of these different integrins, and each integrin can
10 bind to more than one laminin isoform [10, 11].

Along with type IV collagens, nidogens and perlecan, glycoproteins of the laminin family are the major constituents of brain microvessel BMs [8, 12, 13]. These BMs have a complex structure and are produced by both endothelial and glial cells [13]. Endothelial cells contribute laminins containing α_4 and α_5 chains
15 to these BMs, whereas glial cells synthesize laminins containing α_1 and α_2 chains [13]. In human brain capillary BMs we have recently observed a weak expression of the α_4 chain-containing laminin-9. Interestingly, during progression of human gliomas, the expression of capillary BM laminins containing α_4 chain switches from the predominant laminin-9 ($\alpha_4\beta_2\gamma_1$) to laminin-8 ($\alpha_4\beta_1\gamma_1$) [5].
20 Laminin-8 and its receptors, integrins $\alpha_3\beta_1$ and $\alpha_6\beta_1$, appear to be important to the functioning of endothelial cell BMs, which play a role in the maintenance of the blood-brain barrier [14, 15]. Recently, the association of the laminin α_4 chain with angiogenesis has been demonstrated *in vivo* and *in vitro* [16]. Some cultured glioma cell lines can also produce α_4 -containing laminins. Laminin-8 is
25 thought to play a role in cell migration during development, wound healing, and angiogenesis [8, 10, 14].

Brief Description of the Figures

Figure 1 shows uptake of Ac-LDL by various cultures and co-cultures; endothelial cells (HBMVEC) are positive (green fluorescence) but glioma cells
30 (M59K) and normal astrocytes (HAST 040) are negative; in co-cultures

HBMVEC+M059K and HBMVEC+HAST040, endothelial cells are positive, whereas other cells are negative (DAPI was used to counterstain cell nuclei (blue fluorescence)).

Figure 2 shows Laminin $\alpha 4$, $\beta 1$, and $\beta 2$ chain expression in cells and
5 conditioned media of pure cultures:

Fig 2A. Immunolocalization of laminin chains in cells where normal brain endothelium (HBMVEC) expresses $\alpha 4$ and $\beta 2$ chains (consistent with laminin-9, $\alpha 4\beta 2\gamma 1$), whereas astrocytes (HAST 040) do not express these laminin chains; M059K glioma cells, however, express $\alpha 4$ and $\beta 1$ chains consistent with laminin-
10 8 ($\alpha 4\beta 1\gamma 1$). Indirect immunofluorescence.

Fig. 2B. Western blot analysis of conditioned media shows that endothelial cells (HBMVEC) secrete chains of laminin-9 ($\alpha 4$ and $\beta 2$), astrocytes (HAST 040) show little to no secretion of any studied chains, and M059K glioma cells secrete chains of laminin-8 ($\alpha 4$ and $\beta 1$) (T98G, lysate of T98G glioma cells expressing
15 laminin-8 chains only ($\alpha 4$ and $\beta 1$) were used as positive control; equal amounts of conditioned media protein were applied to each lane. Note complete agreement between the results of immunostaining (Fig. 2A) and Western blotting (Fig. 2B)).

Figure 3 shows the Laminin $\alpha 4$, $\beta 1$, and $\beta 2$ chain staining of co-cultures;
20 live co-cultures were exposed to Ac-LDL (green color, to reveal endothelial cells) and then fixed and simultaneously stained for select laminin chains (red color) and nuclei (DAPI, blue color); in endothelial-astrocyte co-cultures (HBMVEC+HAST040) $\alpha 4$ and $\beta 2$ chains are expressed in Ac-LDL-positive endothelial cells only but not in Ac-LDL-negative astrocytes (arrows); $\beta 1$ chain is
25 largely absent; in endothelial-glioma co-cultures (HBMVEC+M059K), $\alpha 4$ chain is expressed by both cell types and $\beta 2$ chain, only by endothelial cells; significantly, $\beta 1$ chain is expressed not only by Ac-LDL-negative glioma cells (arrowheads) but also by Ac-LDL-positive endothelial cells.

Figure 4 shows the Cell viability assay; viability of glioma cell lines M059K
30 and U-87MG as well as of normal endothelial cell line HBMVEC after treatment

with Morpholinos sense or antisense oligos and delivery factor is higher than 90%; no significant difference from parallel untreated control cultures was detected with any treatment (cell viability without treatment was taken as 100% and cell numbers were determined using MTS assay).

5 Figure 5 shows indirect immunofluorescence Laminin $\alpha 4$ and $\beta 1$ staining of antisense-treated co-cultures; co-cultures of M059K or U-87MG with HBMVEC treated with sense oligos to laminin $\alpha 4$ and $\beta 1$ chains for 5 days, and the patterns of laminin chain expression are similar to untreated cultures (upper row, cf. Fig. 3), whereas treatment with antisense oligos to either laminin $\alpha 4$
10 (antisense $\alpha 4$) or laminin $\beta 1$ (antisense $\beta 1$) chain partially inhibits both $\alpha 4$ and $\beta 1$ chain expression (middle rows); finally, treatment with antisense oligos for both chains (antisense $\alpha 4 + \beta 1$) abolishes staining (lower row).

 Figure 6 shows Western blot analysis of laminin-8 $\alpha 4$ and $\beta 1$ chains in conditioned media of co-cultured M059K and HBMVEC cells where incubation
15 with Morpholino sense and antisense oligos was for 3 or 6 days.

Fig. 6A, a 200-kDa band corresponding to laminin $\alpha 4$ chain in co-culture on days 3 and 6, and the amount of immunoreactive $\alpha 4$ laminin was diminished by antisense oligos to either $\alpha 4$ or $\beta 1$ or, especially, $\alpha 4 + \beta 1$.

Fig. 6B, a 230-kDa band corresponding to laminin $\beta 1$ chain in co-cultures on
20 days 3 and 6, and the combination of antisense oligos ($\alpha 4 + \beta 1$) was efficient in decreasing the amount of immunoreactive $\beta 1$ chain band at both time points.

Figs 6C and 6D, Western blots of fibronectin (240 kDa band) on day 6 after stripping the respective membranes from $\alpha 4$ and $\beta 1$ chain detection and reprobing them for fibronectin (these lanes are shown for loading control
25 purpose), and only human (but not serum) fibronectin was detected by this antibody: *Lane 1*, sense oligos for $\alpha 4 + \beta 1$ chains; *Lane 2*, antisense oligo for $\alpha 4$ chain; *Lane 3*, antisense oligo for $\beta 1$ chain; *Lane 4*, antisense oligos for $\alpha 4 + \beta 1$ chains.

Figure 7 shows measurement of invasion in co-cultures after antisense

treatment using the Matrigel invasion assay which demonstrates a significant decrease in the fraction of cells that invaded through Matrigel in antisense-treated cultures (an even more pronounced effect is seen with a combination of antisense oligos; similar results were obtained with M059K and U-87MG glioma cell lines; *, $p < 0.04$; **, $p < 0.001$ by ANOVA with invasion in sense-treated cultures was taken as 100%).

Detailed Description of the Invention

Since laminin-8 appears to be associated with GBM recurrence *in vivo*, we hypothesized that it might play a role in tumor invasion. Because of the complexity of *in vivo* experiments, we first explored this possibility *in vitro* using single cultures and co-cultures of brain microvascular endothelial cells, normal fetal brain astrocytes and several GBMs. We sought to analyze whether the patterns of laminin chain expression in cell culture would be similar to those seen in normal brain and in gliomas, and whether inhibition of laminin-8 expression by an antisense approach would alter glioma invasiveness through a reconstituted BM (Matrigel).

Antisense oligonucleotides (oligos) that bind and inactivate specific RNA sequences may be the best tools for studying gene function, regulation of gene expression, and interactions between gene products. Highly specific antisense oligos that mimic the DNA template for RNA production are used to bind to the complementary RNA and to prevent protein translation [17,18]. Antisense oligos are the fastest, simplest and most cost effective tools for testing new therapeutic targets for drug development. The antisense approach was used in our present study to inhibit the expression of laminin-8 in cell culture.

Our results show that normal cultured astrocytes and endothelial cells mostly express laminin-9 as seen in normal brain tissue. Glioma cells predominantly express laminin-8, again similar to the *in vivo* situation. Most importantly, antisense blocking of laminin-8 chain expression resulted in the inhibition of glioma invasion through Matrigel. These data show that laminin-8 is an important for glioma invasion and an effective target for antitumor therapy.

That is, the differences in laminin-8 chain expression are not merely indicators of the malignant cells but are actually linked to invasiveness of the malignancy.

Co-Culture of Gliomas, Astrocytes and Brain Endothelial Cell Lines.

Two types of human GBM cell lines (M059K and U-87MG; from ATCC, Rockville, MD), a normal human brain microvascular endothelial cell line (HBMVEC, obtained from Dr. Ken Samoto, Japan), and normal human fetal brain astrocytes HAST 040 (from Clonexpress, Inc., Gaithersburg, MD) were used. U-87MG cells were cultured in Eagle's MEM with 10% fetal calf serum (FCS), L-glutamine, sodium bicarbonate, non-essential amino acids, antibiotics, and sodium pyruvate. M059K cell line was maintained in DMEM/F12 medium, FCS, supplements and antibiotics as above. The HAST 040 cell line was cultured in 50:50 DMEM/F12 supplemented with 5% FCS and antibiotics (25 µg/ml of gentamycin and 2.5 µg/ml of fungizone) during regular maintenance of astrocytes. The medium was replaced with fresh medium every third day to maintain optimal growth. HBMVEC were cultured in RPMI 1640 medium with 10% FCS, 10% NU-serum, sodium pyruvate, L-glutamine, non-essential amino acids, and antibiotics. Cell lines were maintained at 37°C in a humidified 5% CO₂ incubator and subcultured with trypsin-EDTA every 3-4 days. Cell lines were co-cultured at a ratio glioma:endothelium of 5:1 in 4-well chambers and examined at different time points (24 h, 3 days, 5 days). Co-cultures of normal human astrocytes HAST 040 and HBMVEC cells were cultured at the same ratio of 5:1 in 4-well chambers and examined at different time points (24 h, 3 days, 5 days).

Antisense Treatment of Glioma-Endothelial Co-Cultures.

Morpholino™ (phosphorodiamidate morpholino oligomer) oligos custom made by Gene Tools, Inc. (St. Louis, MO) for laminin α₄ and β1 chains were as follows:

α₄ antisense 5'AGCTCAAAGCCATTTCTCCGCTGAC3',

α₄ sense 5'GTCAGCGGAGAAATGGCTTTGAGCT3';

β1 antisense 5'CTAGCAACTGGAGAAGCCCCATGCC3';

β1 sense 5'GGCATGGGGCTTCTCCAGTTGCTAG3'.

- Gene Tools protocol was used according to company recommendations. The new Special Delivery Formulation consisted of a pre-paired duplex of Morpholino oligo and partially complementary DNA oligo, together with a weakly basic delivery reagent, ethoxylated polyethylenimine (EPEI). Morpholino oligos are stable and totally nuclease-resistant so there is no need for re-delivery. Co-cultures of glioma cells with normal brain endothelium were treated with anti-sense oligos to laminin-8 chains, $\alpha 4$ and $\beta 1$, for select time intervals (3 and 6 days), alone or in combination. To make the delivery mixture, 0.5 mM antisense $\alpha 4$ or $\beta 1$ laminin chain or 0.5 mM sense oligos (negative control) Morpholino/DNA stock solution (Gene Tools) were added to H₂O and mixed. Two hundred μ M EPEI Special Delivery solution was added, vortexed and incubated at room temperature for 20 min. to generate the complete delivery solution. Medium was removed from a 24-hr co-culture and the solution with a specific oligo in fresh medium was added to cells, and placed into a CO₂ incubator. After 3 hrs, delivery solution was aspirated and replaced with fresh serum-containing medium. Medium was changed every 2 days. Each oligo was assessed at 4 incubation time points: 2, 4, 6 and 8 days (co-culture time being 3, 5, 7, and 9 days, respectively). Another set of controls included endothelial or glioma cells alone.
- Immunohistochemistry.** Cells were incubated in culture with or without Morpholino and at select time periods were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and immunostained for laminin chains and endothelial cell markers. These markers included von Willebrand factor (Sigma Chemical Co., St. Louis, MO), CD31 (clone HC1/6, Cymbus Biotechnology/Chemicon International, Temecula, CA, and clone JC70A, Dako, Carpinteria, CA), CD34 (clone QBEnd 10, Dako), and CD105 (clone P3D1, Chemicon). Uptake of Alexa Fluor 488-labeled acetylated low-density lipoprotein (Ac-LDL, Molecular Probes, Eugene, OR) was also used to identify endothelial cells. Briefly, cells were incubated for 24 hr in medium with 5 μ g/ml labeled Ac-LDL, then washed, fixed and permeabilized. Cells were then counterstained with 10 ng/ml 4', 6-diamidino-2-phenylindole (DAPI, Sigma) to visualize nuclei and additionally immunostained for select laminin chains. Primary monoclonal (mAb)

and polyclonal (pAb) antibodies were used to the α 4 laminin chain (mAb FC10 [19], and pAb 377 [5]), β 1 laminin chain (mAb LT3; Upstate Biotechnology, Lake Placid, NY), and β 2 laminin chain (mAb C4 obtained from the Developmental Studies Hybridoma Bank, Department of Biology, University of Iowa, Iowa City, IA).

Western Blot Analysis. Serum-free conditioned medium was obtained from the same number of cells in the same volume of medium from the co-cultures that were cultured for the same period of time. Conditioned media from co-cultures were concentrated 10-fold by filtering through Centriplus filtration devices (Millipore, Bedford, MA) and proteins were separated using 3-8% gradient Tris-acetate SDS-PAGE (Invitrogen, Carlsbad, CA) under reducing conditions. Lysates of human glioma T98G, known to express laminin-8 [15], were used as a positive control. The gels were blotted onto nitrocellulose membrane (Invitrogen, Carlsbad, CA). The membranes were probed with mAbs followed by chemiluminescent detection using the Immune-Star kit with alkaline phosphatase-conjugated secondary antibodies (Bio-Rad, Hercules, CA). Antibodies were used to the laminin α 4 chain (mAb 8B12 [15]) and β 1 chain (mAb LT3). Antibody to fibronectin 8th type III repeat (mAb 568 [20]) was used to control for equal loading of gel lanes.

Cell Viability Assay. Cell numbers were measured with the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI). It was designed for the determination of the number of viable cells using MTS dye [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]. According to the manufacturer's instructions, a small amount of the CellTiter 96® AQueous One Solution Reagent was added directly to culture wells, and after 3 hours of incubation the absorbance at 490 nm was recorded using an ELISA reader, Spectra Max Plus 384 (Molecular Devices, Sunnyvale, CA). The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. All cell lines were treated exactly as described above in the section "Antisense Treatment of Glioma-Endothelial Co-Cultures". For viability assay, cells were incubated after treatment with Morpholino sense and antisense oligos

and/or delivery factor for three days, the average time point that was used in our experiments. Each experiment was performed in triplicate and was repeated twice.

In Vitro Invasion Assay. Invasion studies were conducted using the Matrigel™ BM matrix assay developed for quantitative measurement of tumor cell invasiveness. Most tested cells characterized as invasive and metastatic *in vivo* are able to invade Matrigel *in vitro* [21,22,23]. We used BioCoat™ Matrigel™ invasion chambers (12-well cell culture inserts containing an 8.0 µm PET membrane with a uniform layer of Matrigel, from Becton Dickinson, Bedford, MA). The coated filters were rehydrated with warm serum-free DMEM (2 ml per chamber). The upper chamber was filled with 2.5×10^4 cells in serum-free medium. The lower chamber was filled with DMEM containing 5% FCS as a chemoattractant towards which the cells migrate. The chambers were incubated for 22 h at 37°C in a 5% CO₂ atmosphere. Cells from the upper surface of the filters were removed by scrubbing with a cotton swab and those migrating to the lower surface of the filters were fixed and stained with hematoxylin and eosin. The number of cells that penetrated the filter was counted in 10 microscopic fields of each filter under x 200 magnification in both experimental and special control membranes using a Zeiss Axiophot microscope connected to an image processing and measuring system (Hamamatsu, Japan). Percent invasion is expressed as mean cell number from invasion chamber to mean cell number from control chamber according to the manufacturer's recommendation. Assays were carried out in triplicates. Four independent experiments were performed for each type of co-culture with each treatment.

Statistical analysis. The data from the cell viability assay and invasion experiments were statistically evaluated by ANOVA test using GraphPad Prism 3 software program (GraphPad Software, San Diego, CA). $P < 0.05$ was considered significant.

Immunohistochemistry of Endothelial Markers and Laminin Chain Expression in Untreated Cultures.

Several endothelial markers were tested in order to select the best one, which might be used to reliably differentiate endothelial cells from normal and

malignant astrocytes in co-cultures. In preliminary experiments, fluorescent Ac-LDL consistently labeled endothelial cells [24] much more uniformly than did antibodies against von Willebrand factor, CD31, CD34 or CD105.

Uptake of fluorescent Ac-LDL was, therefore, used to identify endothelial cells in subsequent experiments with co-cultures. In pure endothelial cultures, most if not all cells displayed predominantly punctate fluorescence with a perinuclear distribution (Fig. 1). Cultures of normal astrocytes and glioma cell lines were largely negative (Fig. 1), although some cells showed low background fluorescence. Ac-LDL uptake allowed identifying positive endothelial cells in co-cultures as well (Fig. 1).

Cultures were then immunostained for chains of laminin-8 and laminin-9. In accordance with the *in vivo* situation, cultured normal endothelial cells stained positive for $\alpha 4$ and $\beta 2$ chains, compatible with the presence of laminin-9 (Fig. 2A). At the same time, staining for laminin-8 $\beta 1$ chain was mostly negative (Fig. 2A). Normal fetal astrocytes did not appreciably stain for any tested laminin chain (Fig. 2A). In contrast, glioma U-87MG (not shown) and M059K cells were positive for laminin-8 $\alpha 4$ and $\beta 1$ chains but largely negative for laminin-9 $\beta 2$ chain (Fig. 2A). These results were fully confirmed by Western blot analysis of conditioned media from cultures with equal protein loading (Fig. 2B).

In co-cultures of normal astrocytes and HBMVEC, mostly $\alpha 4$ and $\beta 2$ chains could be seen, with very little $\beta 1$ chain expression (Fig. 3). However, in co-cultures of glioma cells with HBMVEC, $\alpha 4$ and $\beta 1$ chains were predominantly expressed (Fig. 3). An important finding was that HBMVEC, when co-cultured with malignant astrocytes, started expressing laminin $\beta 1$ chain, in contrast with its absence in endothelial cells alone or in co-culture with normal astrocytes (Fig. 3).

These data show that co-cultures of normal astrocytes and endothelial cells mostly expressed laminin-9 in accordance with our previous *in vivo* results [5]. Furthermore, similar to the *in vivo* situation, glioma cells alone and in co-cultures with endothelial cells mostly expressed laminin-8. Therefore, the established co-culture system resembled the situation *in vivo* in both a normal

and a tumor brain environment. The laminin expression data thus strongly support the hypothesis that glioma-endothelial co-cultures is a valid model to study further the inhibition of expression of laminin-8 as a new glioma marker associated with tumor progression and recurrence development.

5 **Cell Viability Assay.** In order to test the potential toxicity of sense and antisense Morpholino oligos and the delivery factor EPEI, cell viability was measured using MTS-based CellTiter 96 assay. The relative numbers of viable cells of three cell lines U-87MG, M059K and HBMVEC, which had been treated with oligos and/or delivery factor, were compared with cell numbers of replicate
10 cultures of corresponding cell lines without any treatment (taken as 100%). Cell viability for each cell line after oligo treatment in two separate experiments was higher than 90% (Fig. 4). This did not differ significantly from untreated controls ($p>0.05$). Based on these data we conclude that Morpholinos oligos and/or delivery factor did not exert any significant toxic effect on any of the cell lines.

15 **Immunohistochemistry of Laminin Chain Expression in Antisense-Treated Cultures.** Since glioma-endothelial co-cultures mostly expressed $\alpha 4$ and $\beta 1$ chains of laminin-8 (but not laminin-9 $\beta 2$ chain), antisense oligos were used only to block laminin-8 expression. Treatment with $\alpha 4$ antisense resulted in markedly decreased staining for this chain and a reduction of staining for the $\beta 1$
20 chain (Fig. 5). A similar result was seen with $\beta 1$ antisense treatment, compatible with the role of this chain in laminin trimer assembly. As shown in the lower row in Fig. 5, a combination of the two oligos dramatically reduced staining for $\alpha 4$ and $\beta 1$ chains at all time points.

Western Blot Analysis of Pure Cultures and Co-Cultures. In lysates of
25 cultures and co-cultured cells, the signals for laminin $\alpha 4$ and $\beta 1$ chains were very weak and detectable only on days 5-7 of culture or co-culture (data not shown). Therefore, the amounts of these chains were further analyzed in conditioned media after their substantial and equal fold concentration and normalization by total protein and fibronectin content.

30 As shown in Fig. 6, both $\alpha 4$ and $\beta 1$ chains could be detected in sense-

treated cultures at days 3-6, as well as in a positive control (T98G glioma cell lysate [15]). Antisense treatment of either chain resulted in a decreased signal for both chains. Again, maximum inhibition for both chains was achieved by a combined $\alpha 4$ + $\beta 1$ antisense treatment in a concentration of 0.25 mM for each oligo (Fig. 6B). These results were in complete agreement with cell immunostaining data.

Figs. 6C and 6D show reprobing of the membranes to detect fibronectin. Only human fibronectin was detected. T98G, cell lysate of a laminin-8 expressing GBM cell line T98G, used as positive control. Very similar results were obtained using co-culture of HBMVEC with cells another glioma line, U-87MG (data not shown).

Matrigel Invasion Assay. Matrigel invasion assay was used to study the influence of antisense oligos to $\alpha 4$ and $\beta 1$ chains of laminin-8 on the invasive parameters of co-cultures. Corresponding sense oligos were used in control chambers. Another set of controls included endothelial or glioma cells alone.

Two glioma cell lines, U-87MG and M059K, alone had, respectively, 91% and 76% of invasion potential with or without treatment with either single or combined sense oligos against $\alpha 4$ and $\beta 1$ chains. HMBVEC cells demonstrated only 11% invasion. Each experiment was repeated three times in triplicate.

In the next set of experiments, co-cultures of glioma and endothelial cells were treated for three days with $\alpha 4$ and $\beta 1$ antisense oligonucleotides, alone or in combination. Each antisense used in this study significantly inhibited invasion of two different co-culture types (Fig. 7). In this study, 842 microscopic fields with a total of 64,276 cells were evaluated. Specific endothelial staining has demonstrated that both endothelial and glioma cells migrated through Matrigel, with clear prevalence of glioma cells (data not shown). Co-cultures treated with sense oligos to the $\alpha 4$ and $\beta 1$ chains of laminin-8 were considered as controls equal to 100%. When co-cultures were treated with $\alpha 4$ antisense oligo, invasion was blocked by 40% for U-87MG (Fig. 7 right; $p < 0.02$ vs. control) and by 41% for M059K (Fig. 7 left; $p < 0.03$) cell lines compared to cultures treated with sense oligos (taken as 100%). $\beta 1$ antisense oligo also blocked the invasion by 40% for

U-87MG ($p < 0.04$) and by about 47% for M059K ($p < 0.001$) co-cultures. When co-cultures were treated with both antisense oligos against $\alpha 4$ and $\beta 1$ chains, invasion was reduced on average by 62% for U-87MG ($p = 0.0005$) and by 53% for M059K ($p < 0.0001$) co-cultures. In two of five experiments, the inhibition
5 exceeded 75% (not shown here).

A combination of $\alpha 4$ + $\beta 1$ antisense was more efficient at blocking laminin expression than $\alpha 4$ or $\beta 1$ antisense in U-87MG cells and almost equal to $\beta 1$ antisense in M059K cells. Interestingly, $\alpha 4$ and $\beta 1$ chain expression was inhibited more efficiently with lower concentrations of antisense oligos (0.25 +
10 0.25 mM) than with higher ones (0.5 + 0.5 mM). This shows that careful optimization of Morpholino oligo concentrations is important for *in vitro* and *in vivo* studies. It is also important to emphasize the fact that only living cells can penetrate the Matrigel in the invasion assay.

Discussion

15 This is the first study to examine the role of laminin-8 in human tumor cell invasion using antisense inhibitors that block synthesis of this complex trimeric protein. We showed that normal brain endothelial cells expressed small amounts of laminin-9 chains, $\alpha 4$ and $\beta 2$. The expression of laminin-8 chain, $\beta 1$, however, was not detected. Normal astrocytes did not express any of these chains. This *in*
20 *vitro* system is similar to *in vivo* normal brain, where there was a low expression of predominantly laminin-9 [5]. At the same time, glioma cells expressed chains of laminin-8 in culture in accordance with our previous *in vivo* data [5]. Moreover, in co-cultures with glioma cells, brain endothelial cells also started expressing laminin $\beta 1$ chain (compatible with laminin-8 production) in agreement with the
25 finding of laminin-8 overexpression in GBM *in vivo* (Fig. 3).

These data clearly show that normal and tumor *in vivo* patterns of $\alpha 4$ chain-containing laminin isoform expression were retained in the culture setting. Therefore, we were able to validate the respective co-cultures for the patterns of laminin chain expression as a system similar to that observed *in vivo*, both in
30 normal brain tissue and during glioma growth. In combination with several new well-characterized proteins associated with glioma progression, such as

tenascin-C, MMP-2 and MMP-9, [5, 12, 25-29], laminin-8 is an important tool for potential diagnosis or treatment of gliomas. Previously, only laminin-5 was shown to play a role in melanoma invasion [30]. Our present data show that "vascular" laminin-8 also plays a significant role in glioma cell invasiveness.

- 5 Since matrix-degrading proteinases are also important for glioma invasion [31], future research should explore whether proteolysis of laminin is required for glioma invasion.

To probe the role of laminin-8 in glioma invasion, we used antisense oligos to block its expression. The potential of antisense is widely recognized,
10 but it remained largely unfulfilled since, until recently, the available oligos suffered from poor specificity, instability, and undesirable non-antisense effects [32,33]. These problems have been largely solved by the new generation of antisense oligos that offer the promise of safe and effective therapeutics for various diseases including cancer [33,34]. The most promising types of oligos
15 are Morpholino and peptide nucleic acid (PNA; they have nucleobases attached to a neutral "peptide-like" backbone) oligos [32,34]. Morpholino oligos function independently of RNase H and are soluble in aqueous solutions. They work well in the presence or absence of serum, are totally resistant to nucleases, and remain intact in culture medium and in cells indefinitely. Morpholino oligos have a
20 high affinity for RNA and efficiently invade even quite stable secondary structures in mRNAs. They have the highest sequence specificity of all antisense types over a very broad concentration range and appear to be free of non-antisense effects [34,35]. They have high activity in a cell-free translation system and can block target protein production in cultured cells [36]. Morpholino are also
25 effective *in vivo* [37]. Given these properties, Morpholino oligos have been chosen here to inhibit the expression of laminin-8 chains. Special experiments have demonstrated that Morpholino treatment did not affect the viability of any cell line used.

Recently, promising data on the use of antisense technology in glioma
30 cells were obtained. The blocking of matrix metalloproteinase-9 reduced the invasiveness of glioma cells *in vitro* [31,38]. Glioma growth *in vitro* and *in vivo* (as xenotransplants in nude mice) could be inhibited by antisense to telomerase [39].

A recent pilot study showed that antisense to the IGF-I receptor induced glioma cell apoptosis and resulted in clinical improvement in patients [40]. Several clinical trials are currently using antisense oligos for the treatment of other cancers [41].

5 To examine the involvement of laminin-8 in glioma invasion, we needed reliable systems where it was possible to quantify invasion rates and to optimize the dosage of antisense laminin oligos. We used a cell culture system to meet these important needs. One could potentially use glioma cultures. To better mimic the *in vivo* situation, however, and because laminin-8 seems to be
10 produced by both glioma and endothelial cells [15, Fig. 3], we needed to combine glioma cells with brain endothelium in a co-culture [44]. In such a situation, endothelial cells can develop capillary-like structures, and this process is faster when endothelial cells are cultured with tumor astrocytes than with normal embryonic brain astrocytes [45]. We hypothesized that in glioma-
15 endothelium co-cultures there would be more laminin-8 produced, and that this laminin might increase glioma invasion in a Matrigel assay. Research into these issues should facilitate both GBM diagnosis and prognosis, and increase survival of brain cancer patients.

 Matrigel invasion assay was developed for quantitative measurement of
20 the invasiveness of tumor cells through a BM matrix. Most tested cells characterized as invasive and metastatic *in vivo* are able *in vitro* to invade Matrigel, which is a BM-like material from the mouse Engelbreth-Holm-Swarm tumor [21,22].

 When glioma-endothelial co-cultures were treated by antisense, the
25 inhibition of invasiveness on Matrigel was 62% for U-87MG+HBMVEC and 53% for M059K+HBMVEC of that seen in the control cells treated with corresponding sense oligonucleotides. In our experiments, $\alpha 4$ and $\beta 1$ expression was inhibited more efficiently with a lower concentration of antisense oligos (0.25 + 0.25 mM) than with a higher concentration (0.5 + 0.5 mM), although no apparent toxicity
30 was noticed at either concentration. These data may be explained by previous findings, where oligonucleotide receptors on membranes of HepG2 cells were

blocked. It was shown that at relatively high oligonucleotide concentrations, these receptors were saturated and the pinocytotic process assumed larger importance [46]. A similar mechanism may occur in our system, which would explain the obtained results.

5 The use of antisense technology offers an effective future tumor treatment because of its efficiency, specificity and ease of delivery to tumor cells [42,43]. This technology is being continuously developed and refined not only for the drug validation and diagnostic purposes but also for the development of future treatments. The present results demonstrate the effectiveness of antisense
10 approach using laminin-8 as a target for treatment of brain gliomas. Reduction of tumor invasion by antisense to laminin-8 slows the growth and spread of aggressive GBMs. In combination with other treatment methods or with blocking of other targets as well (EGFR, MMPs) it prolongs disease-free periods and increases survival of glioma patients. Laminin-8 blocking for therapeutic
15 purposes may also include the use of specific monoclonal antibodies and/or small interfering RNA (siRNA) and or other drugs specific to Laminin-8 production.

 It remains to be established how laminin-8 promotes glioma invasiveness. One possible mechanism may be stimulation of cell migration. It was previously
20 shown that at least one form of laminin-8 containing $\alpha 4A$ splice variant rather weakly supported cell adhesion and spreading compared to laminin-5 or laminin 10/11 [15, 47]. At the same time, laminin-8 stimulated cell migration better than several other laminin isoforms [15]. Increased expression of laminin-8 in both glioma cells and glioma-adjacent capillary endothelial cells [5, 15, this report]
25 may reduce glial cell adhesion and enhance migration, which is necessary for local tumor invasiveness.

 Many alterations and modifications may be made by those having ordinary skill in the art. Therefore, it must be understood that the illustrated embodiment has been set forth only for the purposes of example and that it
30 should not be taken as limiting the invention as defined by the following claims. The words used in this specification to describe the invention and its various

embodiments are to be understood not only in the sense of their commonly defined meanings, but to include by special definition in this specification structure, material or acts beyond the scope of the commonly defined meanings. Thus if an element can be understood in the context of this specification as
5 including more than one meaning, then its use in a claim must be understood as being generic to all possible meanings supported by the specification and by the word itself. In addition to the equivalents of the claimed elements, obvious substitutions now or later known to one with ordinary skill in the art are defined to be within the scope of the defined elements. The claims are thus to be
10 understood to include what is specifically illustrated and described above, what is conceptually equivalent, and what can be obviously substituted.

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Claims

What is claimed is:

1. A method for reducing invasiveness of a human glioma comprising the step of contacting said glioma with a composition that inhibits
5 expression of Laminin-8 by said glioma.
2. The method according to claim 1, wherein Laminin-8 expression is inhibited by inhibiting the expression of Laminin α 4 chain.
3. The method according to claim 1, wherein Laminin-8 expression is inhibited by inhibiting the expression of Laminin β 1 chain.
- 10 4. The method according to claim 1, wherein Laminin-8 expression is inhibited by inhibiting the expression of both Laminin α 4 chain and Laminin β 1 chain.
5. The method according to claim 1, wherein the composition includes a composition selected from the group consisting of an antisense
15 polynucleotide, a monoclonal antibody and a small interfering RNA.
6. The method according to claim 5, wherein said polynucleotide is a morpholino polynucleotide.
7. The method according to claim 5, wherein the antisense polynucleotide includes an antisense of Laminin α 4 chain.
- 20 8. The method according to claim 7, wherein the antisense of Laminin α 4 chain comprises the 5' to 3' sequence A G C T C A A A G C C A T T
T C T C C G C T G A C.
9. The method according to claim 5, wherein the antisense polynucleotide includes an antisense of Laminin β 1 chain.

10. The method according to claim 9, wherein the antisense of Laminin β 1 chain comprises the 5' to 3' sequence C T A G C A A C T G G A G A A G C C C C A T G C C.

5 11. The method according to claim 5, wherein the antisense polynucleotide includes both an antisense of Laminin α 4 chain and an antisense of Laminin β 1 chain.

12. The method according to claim 11, wherein the antisense polynucleotide includes polynucleotides comprising 5' to 3' sequences of C T A G C A A C T G G A G A A G C C C C A T G C C and A G C T C A A A G C C A
10 T T T C T C C G C T G A C.

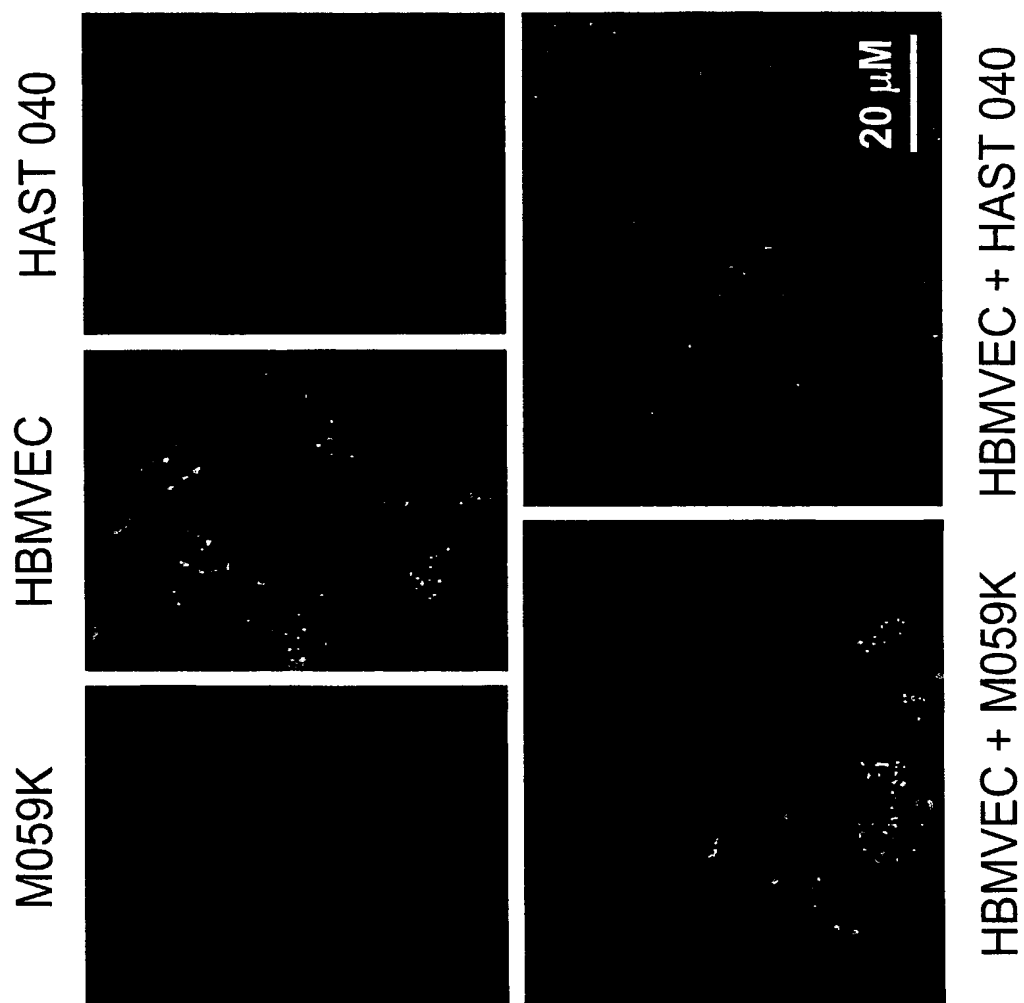


Fig. 1

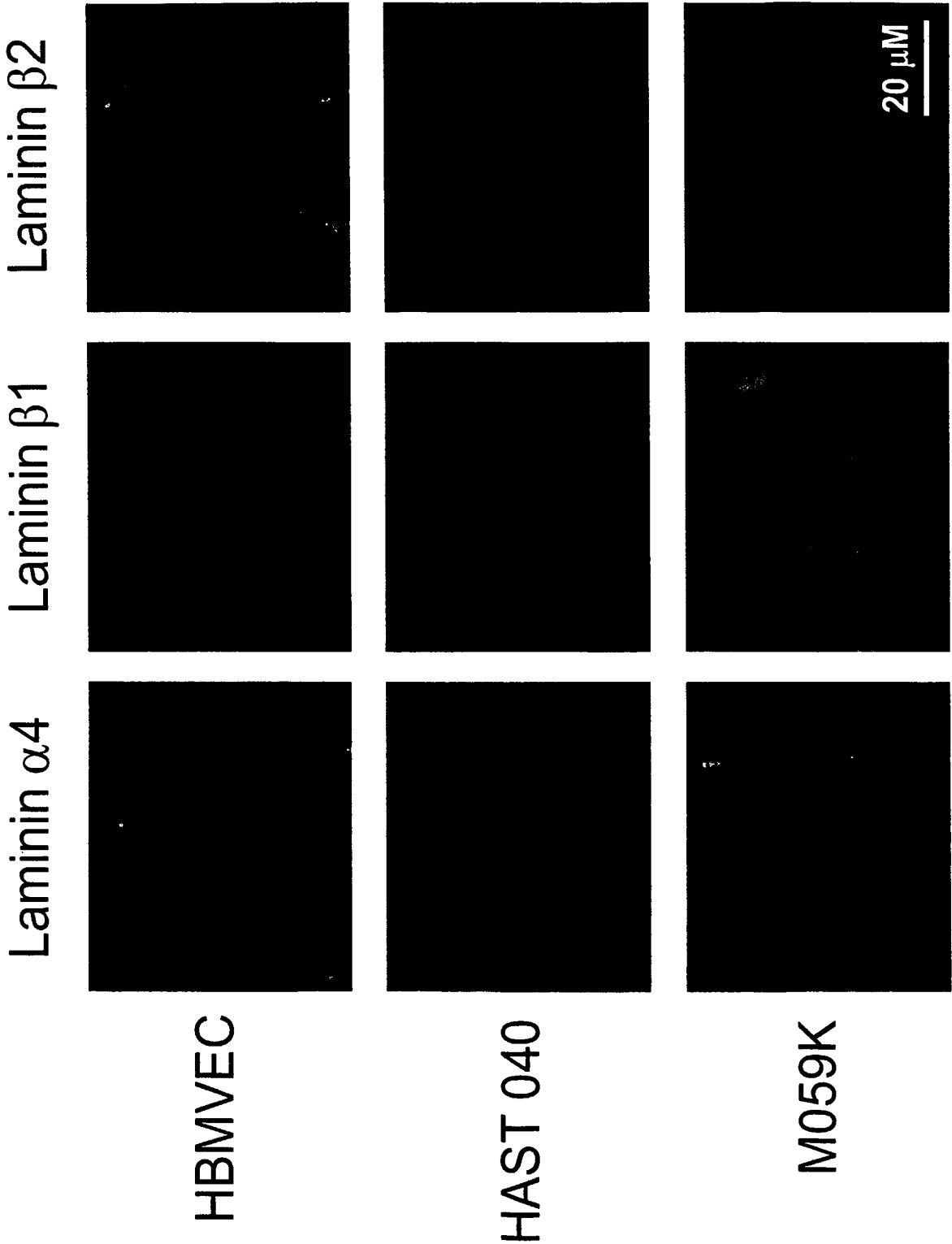


Fig. 2a

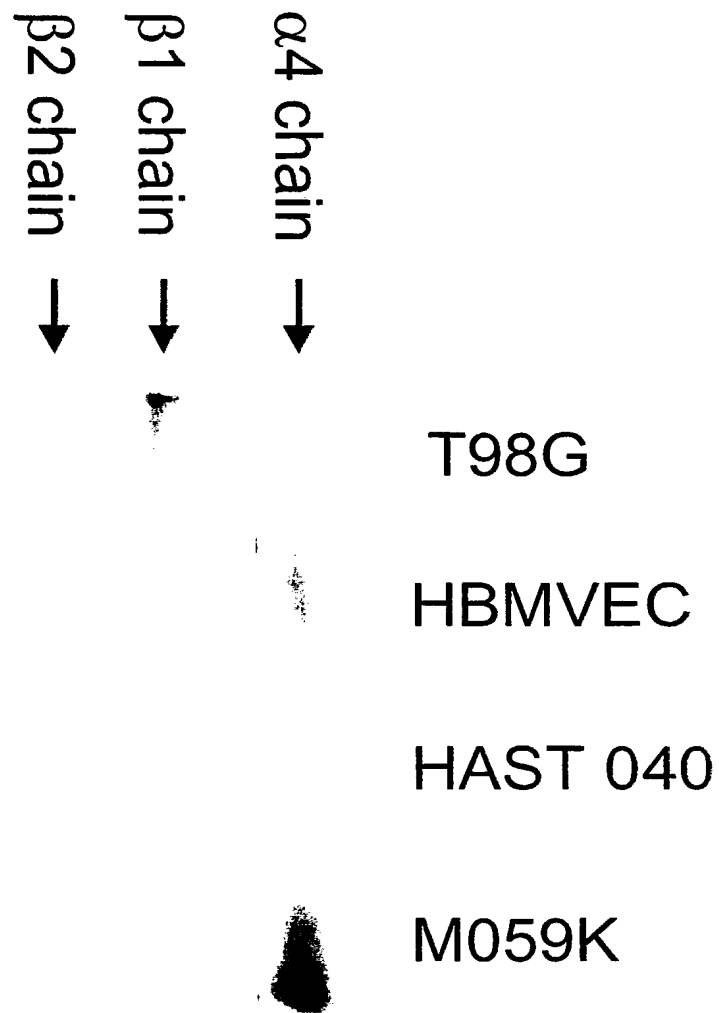


Fig. 2b

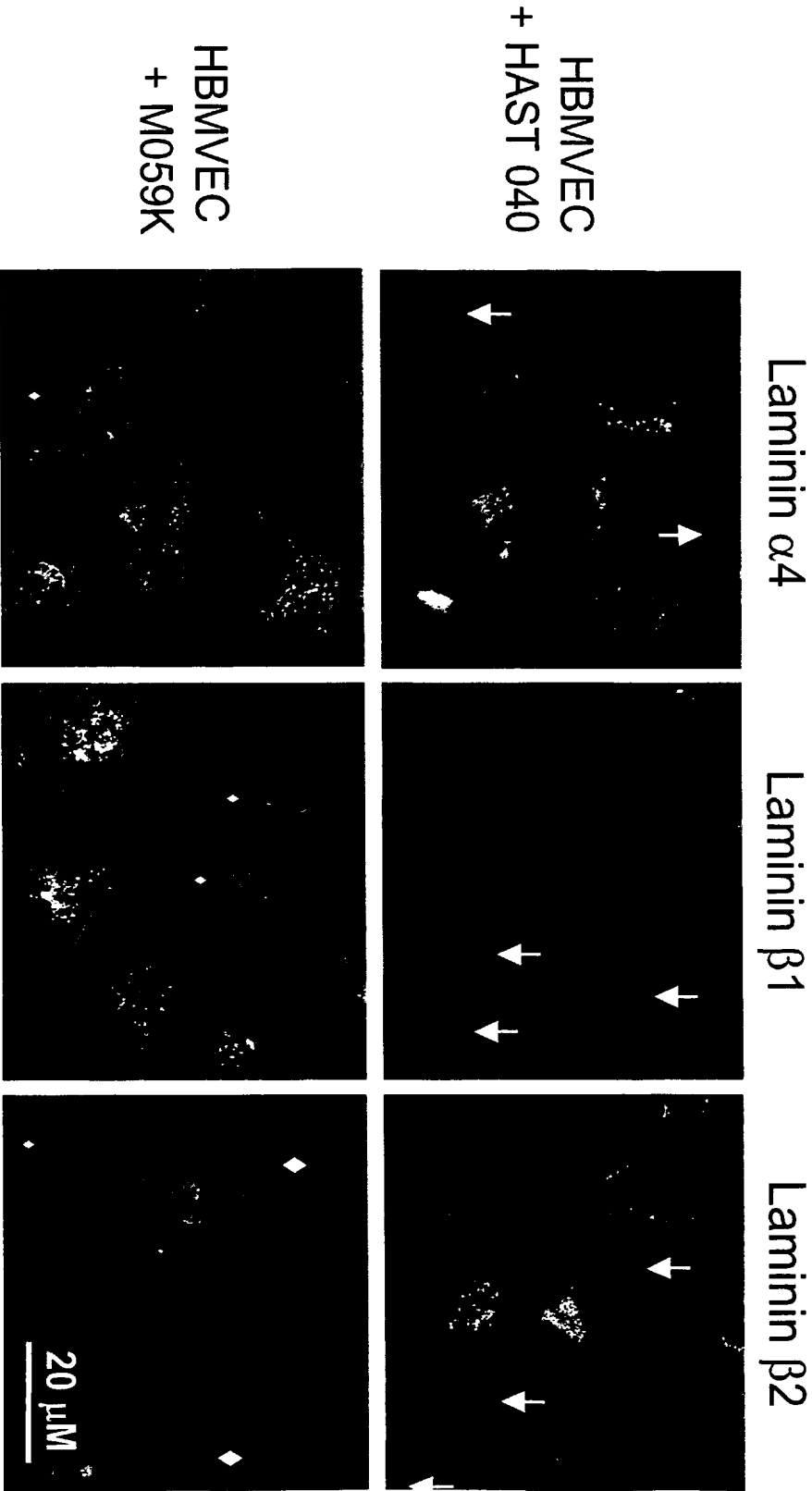


Fig. 3

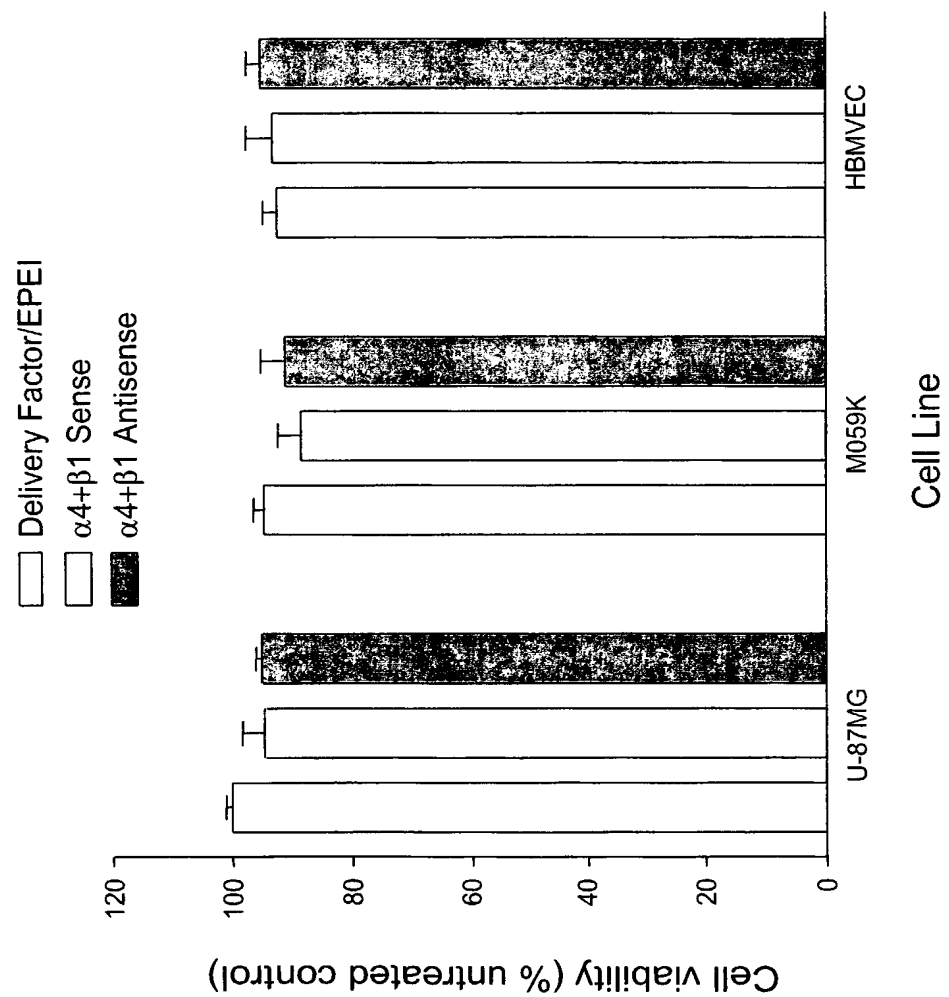


Fig. 4

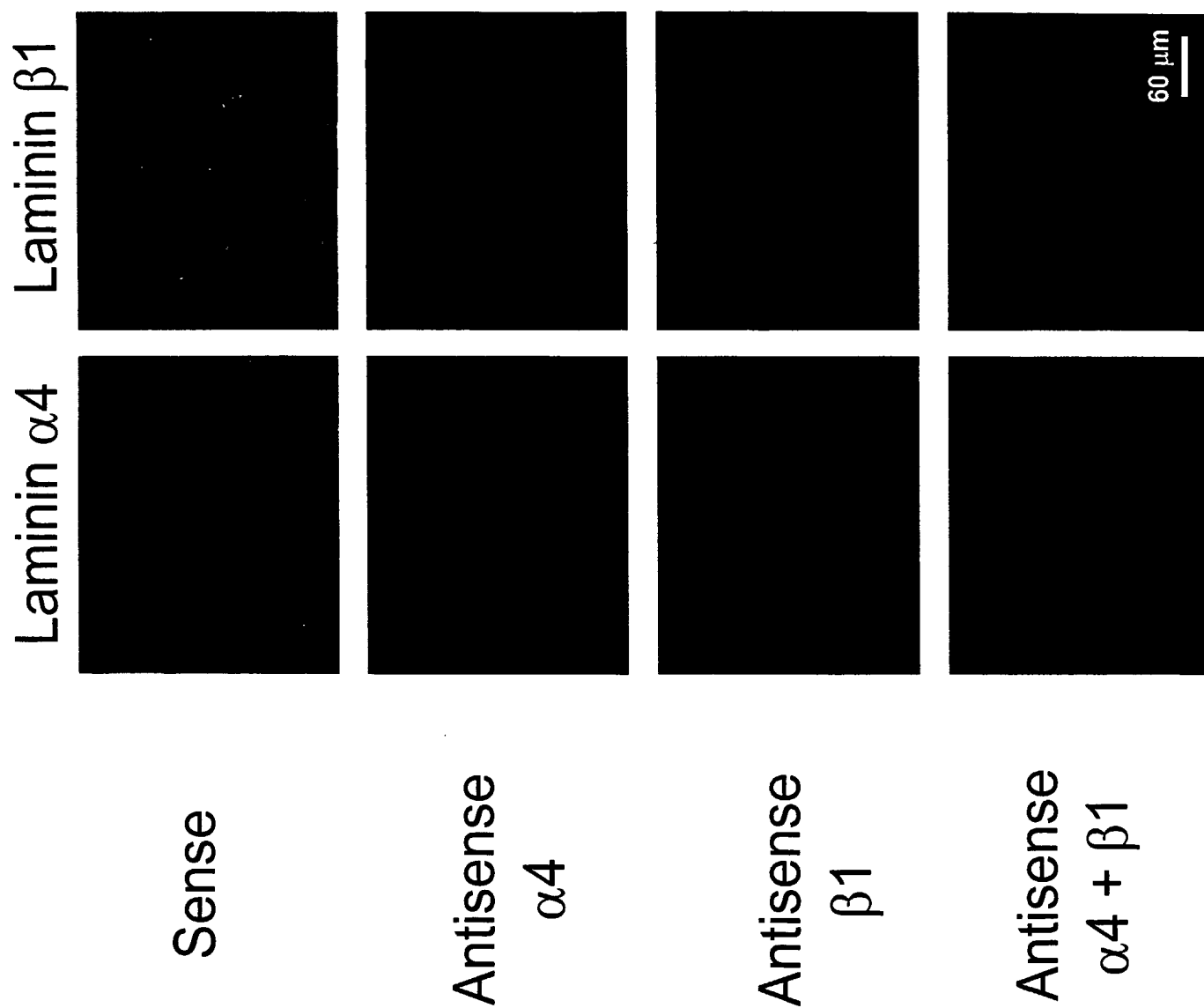


Fig. 5

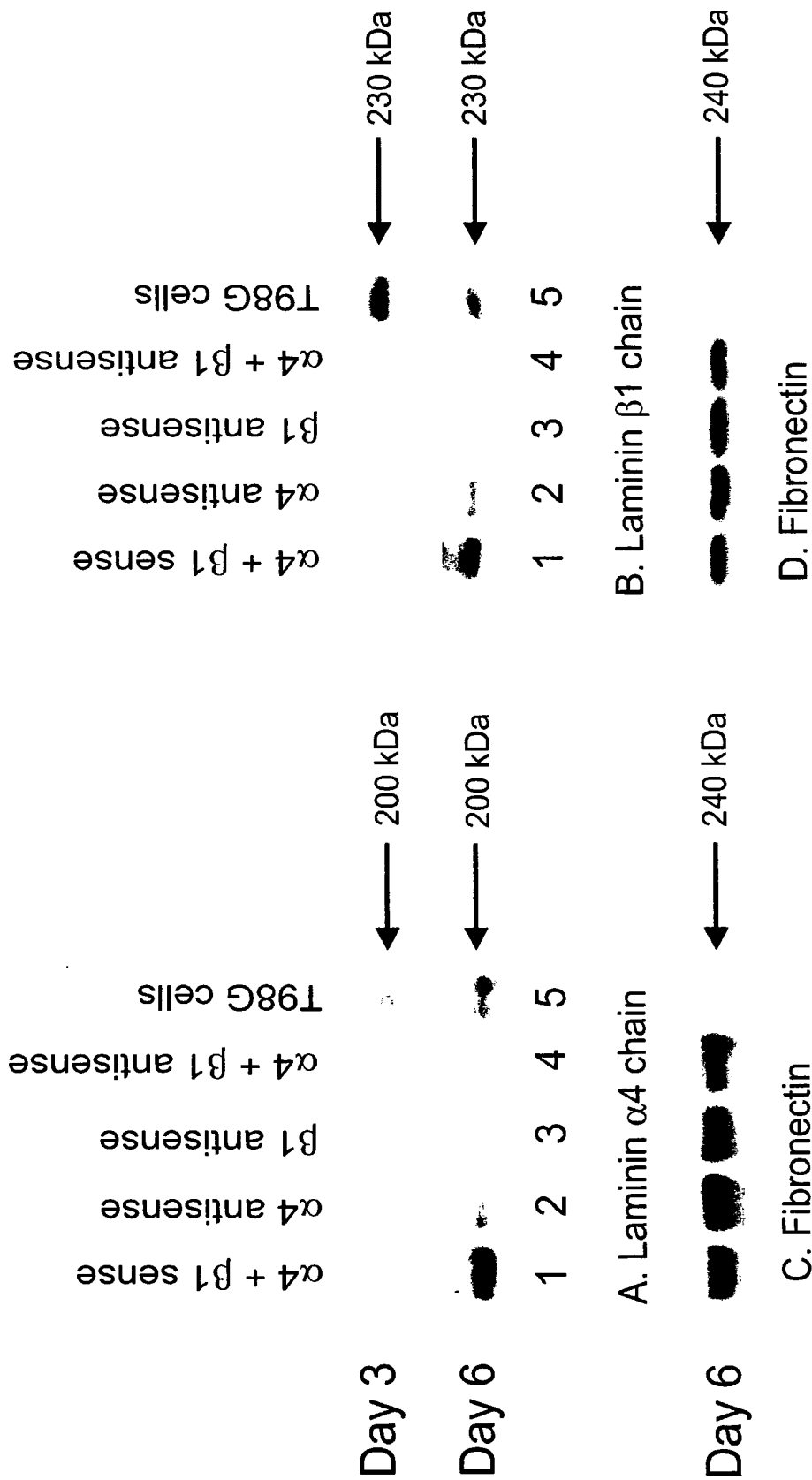


Fig. 6

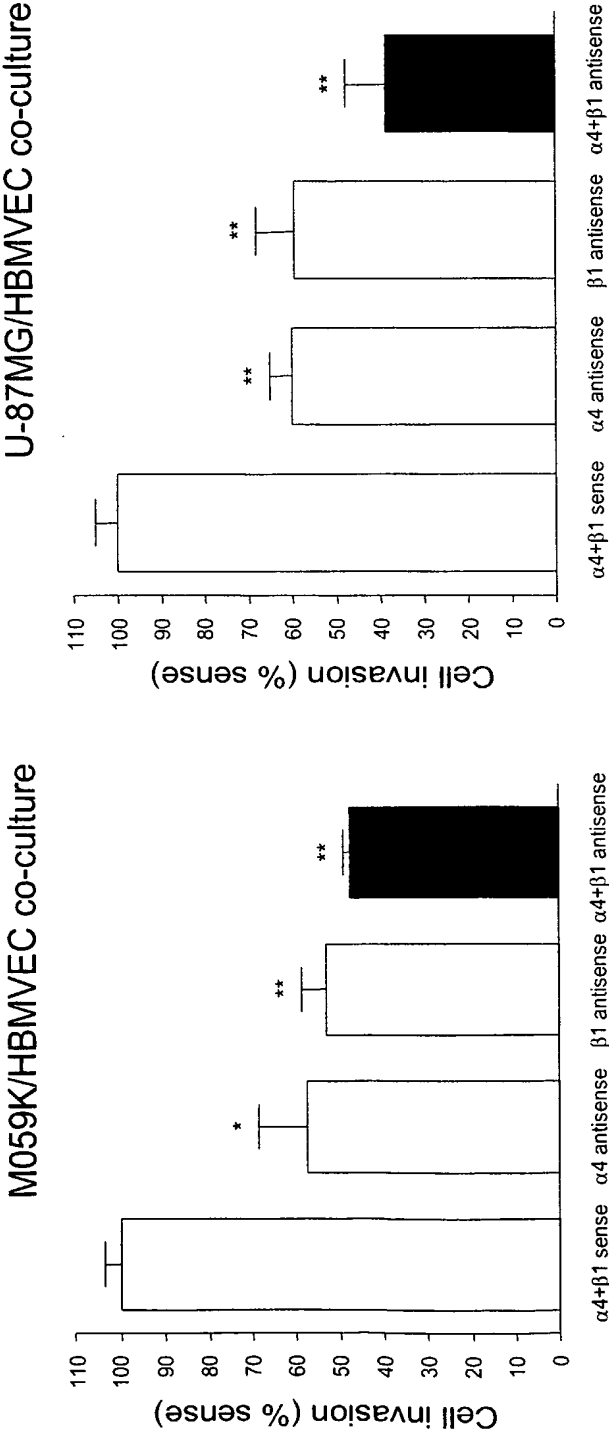


Fig. 7

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ning of each regular issue of the PCT Gazette.

(54) Title: ANTISENSE INHIBITION OF LAMININ-8 EXPRESSION TO INHIBIT HUMAN GLIOMAS

(57) Abstract: Using gene array technology, we observed that an increase of the $\alpha 4$ chain- containing Laminin-8 correlated with poor prognosis for patients with brain gliomas. We established that inhibition of Laminin-8 expression by a new generation of highly specific and stable antisense oligonucleotides (*Morpholino*TM) against chains of Laminin-8 could slow or stop the spread of glioma. This was demonstrated in an *in vitro* model using human glioblastoma multiforme cell lines M059K and U-87MG co-cultured with normal human brain microvascular endothelial cells (HBMVEC). Using Western blot analysis and immunohistochemistry, we con-
firmed that antisense treatment effectively blocked laminin-8 protein synthesis. Antisense oligonucleotides against both $\alpha 4$ and $\beta 1$ chains of laminin-8 blocked significantly the invasion of co-cultures through Matrigel. The results show that Laminin-8 may not only contribute to glioma progression and recurrence as part of the neovascularization process but also by directly increasing the invasive potential of tumor cells.



WO 2005/028617 A3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/29956

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/68; A01N 43/04; C07H 21/04; A61K 31/07
US CL : 435/6, 91.1, 325, 375; 536/24.3, 24.33, 24.5; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 325, 375; 536/24.3, 24.33, 24.5; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THYBOLL et al. Deletion of the laminin alpha 4 chain leads to impaired microvessel maturation. Molecular and Cellular Biology, 2002 Vol. 22:1194-1202, see page 1195, first column, materials and methods.	1, 2, 3, and 5
X	PETAJANIEMI et al. Localizaation of laminin alpha 4 chin in developing and adult human tissues. Journal of Histochemistry and Cytochemistry, 2002 Vol. 50:1113-1130, see page 1114, first column at monoclonal antibodies to human laminin alpha 4 chain.	1, 2, 3, and 5
X	VIRTANEN et al. Distinct changes in the laminin composition of basement membranes in human seminiferous tubules during development and degeneration. Am J Pathol, 1997 Vol. 150:1421-1431.	1, 2, 3, and 5
A	JEN et al. Suppression of Gene Expression by Targeted Disruption of Messenger RNA: Available Options and Current Strategies, 2000 Vol 18:307-319.	1-12
A	BRANCH, AD. A good antisense molecule is hard to find. TIBS, 1998 Vol. 23:45-50, see entire article.	1-12
A	AGRAWAL et al. Antisense therapeutics: is it as simple as complementary base recognition? Molecular Medicine Today, 2000 Vol. 61:72-80.	1-12

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search

13 March 2005 (13.03.2005)

Date of mailing of the international search report

19 APR 2005

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No. **PCT/US04/29956**

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KHAZENZON et al. Antisense inhibition of laminin-8 expression reduces invasion of human gliomas in vitro. Molecular Cancer Therapeutics, 2003 Vol. 2:985-994.	1-12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/29956

Continuation of B. FIELDS SEARCHED Item 3:

Medline, CaPlus, EmBase, CancerLit, WEST, STN sequence

search terms: laminin-8 inhibition, laminin alpha 4, laminin beta, antisense, ribozyme, antibody